Individuals in an endemic region for *Leishmania braziliensis* display lower levels of CD45RO in T cells

Indivíduos em região endêmica para *Leishmania braziliensis* exibem níveis mais baixos de CD45RO em células T

Los individuos en una región endémica para *Leishmania braziliensis* muestran niveles más bajos de CD45RO en las células T

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Abstract
Cutaneous Leishmaniasis (CL) is an important neglected tropical disease, that reaches about 900 thousand to 1.5 million people annually, mainly in Brazil. The present study aimed to characterize these lymphocyte populations and the expression of the CD45RO marker profile in patients with CL. We evaluated CD4+, CD8+, double-positive (DP) and double-negative (DN) T cells and their expression of CD45RO in Cutaneous Leishmaniasis patients, divided into three groups: before treatment (BT), posttreatment (PT) and subclinical (SC). CD4+ T cells had a higher percentage in PT and SC groups when compared to the control group (CT). CD4+ T cells presented a greater expression of CD45RO in BT and PT groups compared to the SC group, that had a lower expression compared with the CT. CD8+ T lymphocytes had a higher percentage in BT when compared with CT and a greater expression of CD45RO when compared to PT, SC and CT groups. We did not observe any statistical significance in the percentage of the DP and DN lymphocytes, however, we noticed that the DP lymphocytes presented a lower expression of CD45RO in the SC group when compared to the CT group, whereas the DN lymphocytes showed a greater expression of CD45RO in the BT group when compared with the other groups. CD4+ T cells appear to be protective in these patients and CD8+ T lymphocytes seem to be associated to pathogenesis, while DP CD45RO+ and DN CD45RO+ lymphocytes appear to play a dual role.
Keywords: Flow cytometry; Leishmaniasis; Cutaneous; Neglected disease, T-Lymphocytes.

Resumo
A Leishmaniose cutânea (CL) é uma importante doença tropical negligenciada, que atinge cerca de 900 mil a 1,5 milhões de pessoas anualmente, principalmente no Brasil. O presente estudo teve como objetivo caracterizar estas populações linfocitárias e a expressão do perfil do marcador CD45RO em pacientes com CL. Avaliamos as células T CD4+, CD8+, duplo positivo (DP) e duplo negativo (DN) e sua expressão do CD45RO em pacientes com

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Leishmaniose cutânea, divididos em três grupos: antes do tratamento (BT), pós-tratamento (PT) e subclínico (SC). As células T CD4+ tinham uma porcentagem maior nos grupos PT e SC quando comparadas ao grupo controle (CT). As células T CD4+ apresentaram uma maior expressão de CD45RO nos grupos BT e PT em comparação com o grupo SC, que teve uma expressão menor em comparação com o grupo CT. Os linfócitos CD8+ T apresentaram uma porcentagem maior em BT quando comparados com CT e uma expressão maior de CD45RO quando comparados com os grupos PT, SC e CT. Não observamos nenhum significado estatístico na porcentagem de linfócitos DP e DN, entretanto, notamos que os linfócitos DP apresentaram uma expressão menor de CD45RO no grupo SC quando comparados com o grupo CT, enquanto os linfócitos DN apresentaram uma expressão maior de CD45RO no grupo BT quando comparados com os outros grupos. As células T CD4+ parecem ser protetoras nestes pacientes e os linfócitos CD8+ T parecem estar associados à patogênese, enquanto os linfócitos DP CD45RO+ e DN CD45RO+ parecem desempenhar um papel duplo.
Palavras-chave: Citometria de fluxo; Leishmaniose; Cutânea; Doença negligenciada; Linfócitos T.

Resumen
La leishmaniasis cutánea (LC) es una importante enfermedad tropical desatendida, que alcanza anualmente entre 900 mil y 1,5 millones de personas, principalmente en Brasil. El presente estudio tuvo como objetivo caracterizar estas poblaciones de linfocitos y la expresión del perfil del marcador CD45RO en pacientes con LC. Se evaluaron las células T CD4+, CD8+, doblemente positivas (DP) y doblemente negativas (DN) y su expresión de CD45RO en pacientes con Leishmaniasis Cutánea, divididos en tres grupos: antes del tratamiento (BT), postratamiento (PT) y subclínico (SC). Las células T CD4+ presentaron un mayor porcentaje en los grupos PT y SC en comparación con el grupo de control (CT). Los linfocitos T CD4+ presentaron una mayor expresión de CD45RO en los grupos BT y PT en comparación con el grupo SC, que tuvo una menor expresión en comparación con el CT. Los linfocitos T CD8+ presentaron un mayor porcentaje en BT en comparación con CT y una mayor expresión de CD45RO en comparación con los grupos PT, SC y CT. No observamos ninguna significación estadística en el porcentaje de los linfocitos DP y DN, sin embargo, notamos que los linfocitos DP presentaron una menor expresión de CD45RO en el grupo SC en comparación con el grupo CT, mientras que los linfocitos DN mostraron una mayor expresión de CD45RO en el grupo BT en comparación con los otros grupos. Los linfocitos T CD4+ parecen ser protectores en estos pacientes y los linfocitos T CD8+ parecen estar asociados a la patogénesis, mientras que los linfocitos DP CD45RO+ y DN CD45RO+ parecen desempeñar un papel doble.
Palabras clave: Citometría de flujo; Leishmaniasis; Cutánea; Enfermedad desatendida; Linfocitos T.

1. Introduction

Leishmaniasis is an important neglected tropical disease, considered a public health issue in more than 105 countries (Organización Panamericana de la Salud, 2019). This disease is found mainly in countries such as East Africa, Southeast Asia, and Latin America, affecting mainly the least favored socioeconomically. In Brazil, in the period between 2015 and 2020, 97,691 cases of LC were reported, of which 1,302 were reported in the state of Pernambuco (Brasil, 2021). In Pernambuco, a state considered endemic to the CL, cases have been reported in all regions of the State.

CL is characterized by the appearance of skin lesions, which can range from one to hundreds. Subclinical patients are characterized by positive diagnosis for *Leishmania*, however, they do not present any clinical manifestations. However, some patients present a positive diagnosis for LC, but do not present the clinical manifestations of the disease, being called subclinical patients. Studies have pointed out that these patients have a decrease in serum levels of cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) and an increase in the levels of interleukin-5 (IL-5), IL-6 and IL-10, suggesting that these patients may modulate the delayed immune response, since they may be constantly exposed to *Leishmania* antigens (Bittar et al, 2007; Follador et al., 2002; Silva et al., 2019). In addition, it has already been seen that monocytes from these patients show a resistance to penetration by *Leishmania braziliensis* (Muniz et al., 2016). Therefore, studies are needed with this population in order to better understand the efficiency of the immune system.

After sand flies inoculation of parasites in the skin, there is an activation of the innate immune system and ultimately an activation of the adaptive immune response, where CD4+ T cells are crucial for healing and/or progression of CL (Brelaz-de-Castro et al., 2012). These T cells are cytokine-producing sources, acting on the mechanisms of healing and pathogenesis of the disease. These lymphocytes may follow two distinct profiles: the Th1 profile, which produces a large amount of IFN-γ that

is essential for the production of nitric oxide (NO), culminating in the death of *Leishmania*; and the Th2 profile, characterized by the production of Interleukin-4 (IL-4) and Interleukin-10 (IL-10), which act to inhibit the production of NO, favoring the multiplication of the parasite (Heinzel et al., 1993; Liew et al., 1990; Murray, 1981; Novais et al., 2013; Scott et al., 1988).

CD8+ T lymphocytes have a double role in the fight against infection. They can act with a lytic function, which will kill the infected cells through the release of granules which contain granzymes and perforins. They may also act in the production of cytokines and chemokines, causing more cells to reach and be activated at the site of infection (Bittar et al., 2007; Ferraz et al., 2017). However, the role of these cells is still controversial. It seems that when the CD8+ T lymphocyte is a cytokine producer, producing cytokines like interferon-γ (IFN-γ), it acts in a protective way, contributing to infection control. However, when this same cell has a cytolytic function, it contributes to the onset/exacerbation of lesions caused by the disease (Koh et al., 2020; Novais et al. 2018).

The double-positive lymphocytes (DP) express on their surface both CD4+ and CD8+ molecules. Its main function is not yet known, but it is believed that these cells develop auxiliary and cytolytic functions (Zuckermann & Husmann, 1996). Studies have shown an increase of these cells in some autoimmune diseases such as atopic dermatitis, autoimmune thyroiditis, in addition to viral infections and cancer (Desfrançois et al., 2009; Iwantani et al., 1993; Nascimbeni et al., 2004).

In contrast, double-negative lymphocytes (DN), appear to exert a regulatory function, as well as a pro-inflammatory function (Fischer et al., 2005). Studies performed by Ferraz et al. (2017) with patients with CL observed that DN lymphocytes were the most found cells in situ when compared to the other cells evaluated by the group. They also showed that these cells, together with the CD4+ T lymphocytes and the NKT, corresponded to 80% of the cytotoxic cells in the lesion. Both DP lymphocytes and DN originate in the thymus. DP cells correspond to 80% of thymocytes, whereas DNs correspond to 5% (Murphy et al., 2014).

The maturation stage of lymphocytes is due to the presentation of peptides of the antigen by Major Histocompatibility Complex (MHC). For activation of CD4+ T cells, it is necessary for MHC to be Class II, while for CD8+ T cells the MHC is Class I (Doyle & Strominger, 1987; Murphy et al., 2014). The evaluation of the activation stage of cells can be done with the CD45RO surface marker along with others, such as CD69, CD25 and/or HLADR. CD45RO is also related to memory cells (Martinez-Arends et al., 1991; Mendes-Aguiar et al., 2016).

The present study aimed to characterize these lymphocyte populations and their and the expression of the CD45RO marker profile in patients with CL. In our study we found a decrease of the CD45RO marker in the DP cells, DN and the CD4+ T lymphocytes. Thus, we contributed to a better knowledge of the immune response in CL. The strength of our study lies in the analysis of human populations and in the staging of the disease process.

2. Methodology

Study population

This is an experimental study. The patients in this study were residents of endemic areas for CL in the state of Pernambuco. The first group, was called control group (CT), composed of healthy individuals living in areas not endemic to the disease (n= 19). A total of 50 patients were included in this study, and they were divided into three groups: a group called subclinical (SC), composed of individuals who presented a positive diagnose clinical, epidemiological and laboratorial (Montenegro Intradermal reaction (MIR) diagnosis for CL but had no lesions of the disease (n= 10); a group before treatment (BT), composed of patients who had not yet started the treatment regimen (n= 33); and a group called post-treatment (PT), composed of patients who had already completed the therapeutic regimen and obtained a clinical cure (n= 7). All patients older than 10 years, who presented clinical, epidemiological and at least two positive laboratory diagnosis, were included in the study. The laboratorial exams, performed by the Reference Service in Leishmaniasis of the Aggeu Magalhães Institute

included: Polymerase chain reaction (PCR), indirect immunofluorescence (IFI), Montenegro Intradermal reaction (MIR), direct research (DR) using the scarified lesion for parasite visualization or aspiration puncture (AP). The present study was approved by the Ethics Committee of the Aggeu Magalhães Institute/ Fiocruz (CAAE: 11083812.7.0000.5190).

Obtaining Peripheral Blood Mononuclear Cells (PBMC)

A total of 18 ml of venous blood was collected from each patient in tubes containing heparin. After collection, the blood was placed in a Falcon tube containing Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden), then the tube was centrifuged at room temperature in 400xg for 30 minutes. After that, the PBMCs were collected and transferred to another tube, which was washed twice and centrifuged with phosphate buffered saline (PBS) at room temperature, at 300xg for 10 minutes. Subsequently, in an aliquot, 10 µl of cells + 90 µl of Trypan blue dye (Sigma, St. Louis, MO) were added, so that the cells were counted, analyzed for their viability and adjusted to their concentration for the experiment (Brelaz-de-Castro et al., 2012).

Flow cytometry

For the analysis of $CD4^+$, $CD8^+$, DP and DN lymphocyte populations and their activation profile, PBMCs (0.5×10^6 cells) were suspended in PBS and labeled with anti-CD3 - FITC (BD Pharmigen, San Jose, CA), CD4 - PEcy-7 (BD Pharmigen, San Jose, CA), CD8 - APC (Immunotools, Friesoythe, Germany) and CD45RO - PE (BD Pharmigen, San Jose, CA) surface antibodies for a period of 20 minutes (all previously titrated). After that time, the cells were washed with the PBS solution, and then resuspended in 200µl PBS. Samples were then analyzed on the FACSCalibur flow cytometer (Becton Dickinson Company, San Jose, USA), > 20.000 events / tube, using the CELLQuestPro™ software (BD Biosciences, San Jose, CA) for data acquisition and FlowJo software (version 7.6.5, Tree Star Inc., USA) for its analysis. For data analysis the quadrants' limits were based on the negative population, antibody titration, as well as controls using all antibodies except one (Fluorescence minus one from - FMO).

Statistical analysis

For statistical analysis, the GraphPad Prism 5.1 (version 5.1, GraphPad Prism Software, La Jolla, CA, USA) software was used. First, the Shapiro-Wilk test was performed to verify the normality of the data. Then, the T-student and Anova tests were performed when the normality assumptions were observed. When not, the non-parametric Mann-Whitney or Kruskal-Wallis tests were used. The results were considered statistically significant when $p < 0.05$.

3. Results and Discussion

Clinical characteristics of patients

The patients in this study came from endemic areas for Cutaneous Leishmaniasis. All patients received an epidemiological, clinical and laboratory diagnosis for CL. The mean age of the patients was 29.36 years in the BT group, 20.57 years in the PT group, 33.8 years in the SC group and 27.89 years in the CT group. In the group before treatment, a highest number of infected men was observed when compared to the number of women (ratio of 4.5: 1). BT patients presented, at the time of the clinical evaluation, ulcerous lesions in uncovered areas of the body, with evolution time ranging from 18 days to 24 months. The mean number of lesions / scars ranged from 1 to 5 in the BT and PT groups (Table 1).

Table 1 – Main characteristics of the study group.

Characteristics	Study group			
	CT	SC	BT	PT
Number os patients	19	10	33	7
Age	27,89 (26-32)	33,8 (11-63)	29,36 (13-71)	20,57 (12-37)
Ratio M/F	9/10	4/6	27/6	3/4
Mean Duration of disease (months)	-	-	2,97	-
Mean number of lesions/ scars	-	-	1,54 (1-5)	3 (1-5)

Subtitle: CT: group control; SC: group subclinical; BT: group before treatment; PT: group posttreatment.
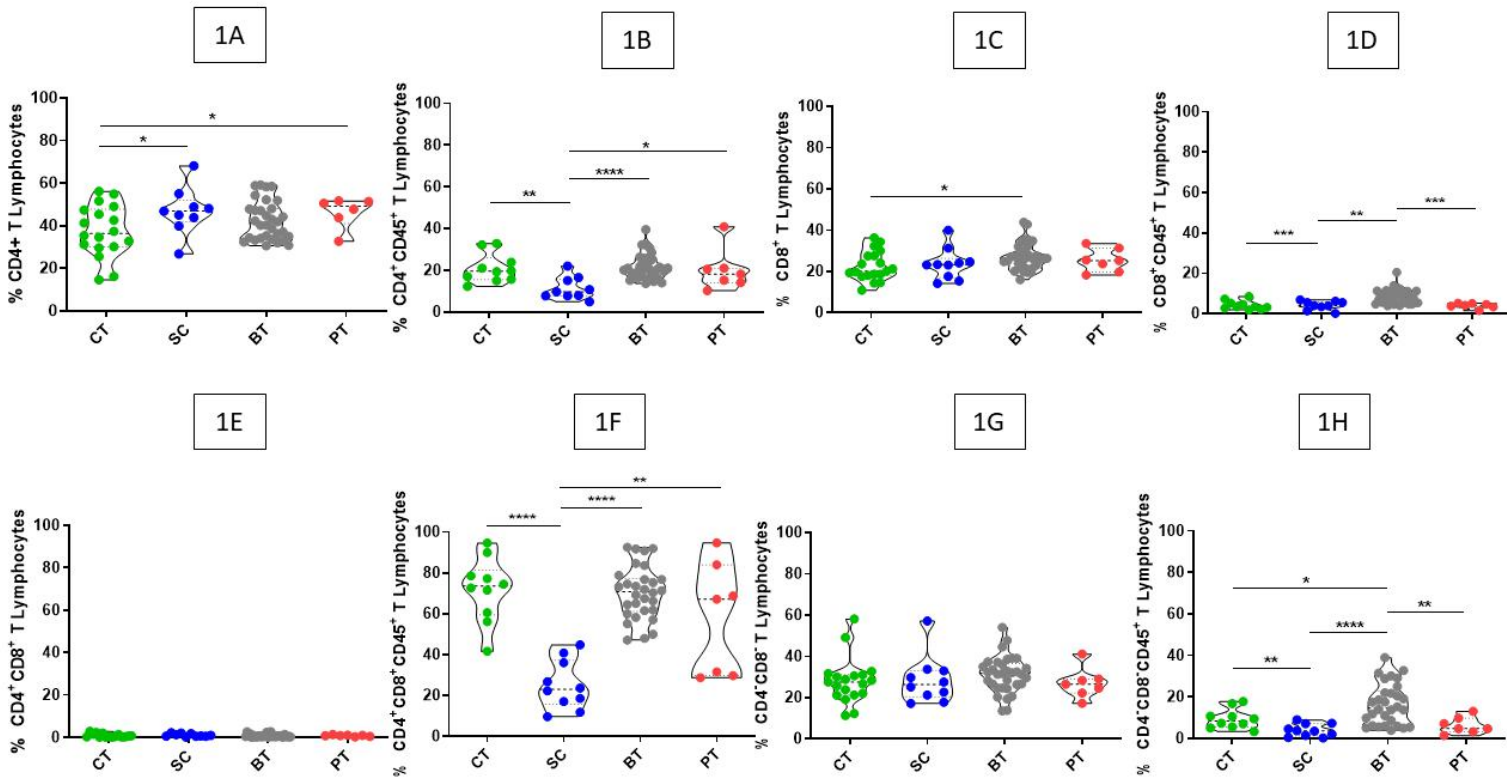Source: Authors.

Immunological evaluation

We observed a statistically significant increase in CD4$^+$ T cells in the PT (p = 0.0431) and SC (p = 0.0311) groups when compared to the control group (Figure 1A). Regarding the expression of CD45RO by CD4$^+$ T cells, we observed that these cells were more positive in the BT groups (p = 0.003) and PT (p = 0.0418), when compared with the SC group. We also observed a decrease in the expression of CD45RO in the SC group (p = 0.0057) when compared to the CT group (Figure1B).

When we evaluated the percentage of CD8$^+$ T lymphocytes (Figure 1C), we observed a statistically significant increase of these cells in the BT group (p = 0.0231) compared to the CT group. Regarding CD45RO expression (Figure 1D), we noticed a greater positivity among BT groups in comparison to PT (p = 0.0007), SC (p = 0.0027) and CT (p = 0.0008).

We did not observe any statistical significance when evaluating the percentage of DP lymphocytes (Figure 1E). When we evaluated their CD45RO positivity (Figure 1F), we observed a greater expression in the BT group compared to SC (p <0.0001), PT when compared to SC (p = 0.0097) and a decrease in their expression in the SC group when confronted with the CT group (p <0.0001).

Finally, when evaluating the DN lymphocytes (Figure 1G), we did not observe any statistical significance. However, when we evaluated the percentage of CD45RO expression of these cells (Figure 1H), we noticed a greater positivity among BT groups when compared to PT (p = 0.0044), SC (p = 0.0001) and CT (p = 0.0473) and a decrease in the SC group when compared to the CT group (p = 0.0089).

Figure 1. A: Percentage of CD4+ T lymphocytes; B: Percentage of activation of CD4+ T lymphocytes; C: Percentage of CD8+ T lymphocytes; D: Percentage of activation of CD8+ T lymphocytes; E: Percentage of DP T lymphocytes; F: Percentage of activation of DP T lymphocytes; G: Percentage of DN T lymphocytes and H: Percentage of activation of DN T lymphocytes in PBMCs of patients with active CL before treatment (BT), posttreatment (PT), subclinical (SC) patients and healthy controls, not carriers of the disease, (CT). Results are expressed as means ± SEM. Differences between groups were considered significant when values of "p" were less than 0.05 and are represented by the "*" symbol.

Source: Authors.

4. Discussion

The results showed an increase in CD4^{+} T cells in the PT and SC groups when compared to the control group. These data corroborate previous findings from our group, where we evaluated PBMCs from patients with CL and observed an increase in these lymphocytes in patients after treatment with chemotherapy (Brelaz-de-Castro et al., 2012). Our data support the idea that these cells are crucial in combating the infection, and consequently, by preventing the infection from progressing.

In addition, we also showed a smaller expression of CD45RO in CD4^{+} T cells in subclinical patients in comparison to the controls, active disease (BT group) and in patients who had completed the therapeutic regimen and obtained clinical cure (PT). This suggests that a greater positivity for this marker may generate a deregulated response, what could favor lesion development, since it may be associated with an increased production of cytokines and consequently with the appearance of lesions. More studies are needed to clarify the role of this marker in CL. Clarêncio et al. (2008) evaluated the percentage of CD4^{+} T cells CD45RO expression in patients with visceral leishmaniasis, before and after treatment, and observed a greater positivity in the posttreatment group when compared to the pre-treatment group. Our study included patients with the tegumentary form, that usually are less aggressive than the visceral form, and that may account for the differences observed.

CD8+ T lymphocytes have been associated with immunoprotection (Brelaz-de-Castro et al., 2012; Cunha et al., 2016), as well as responsible for lesions development (Ferraz et al., 2017; Santos et al., 2013). The latter is associated to its cytokine-producing activity. Some studies indicate that CD8$^+$ T lymphocytes act by destroying macrophages that are infected with *Leishmania*, thus inducing the infection control (Conceição-Silva et al., 1994; Da-Cruz et al., 1994). In our study, these cells presented a higher percentage and expression of CD45RO in the BT group when compared with the other groups studied. These findings suggest that these cells may be involved with the onset of lesions, since the same result was not seen in PT patients.

Double-positive lymphocytes, which carry CD4$^+$ and CD8+ markers on their surface, have been associated with suppressor and cytotoxic roles (Overgaard et al., 2015). These cells have already been observed in lesions of multiple sclerosis, where they have a regulatory function and in HIV infection, which had a high proliferative and effector capacity (Eljaafari et al., 2012; Frahm et al., 2012). In the present study, no statistical significance was obtained in relation to the percentage of these cells between the studied groups. However, as for CD45RO expression, an increase of this marker was seen in the group with active infection (BT) and in the group that obtained clinical cure (PT) and a decrease in the expression of CD45RO was observed in the SC group, suggesting that these cells may have a dual role. However, additional studies with other markers are needed to accurately define their role in CL.

Double-negative lymphocytes (DN) have also been associated with a cytotoxic role. Ferraz et al. (2017) have associated them as one of the main sources of cytotoxic activity at *in situ* lesions in CL, thus contributing to the onset of lesions. It is postulated that these cells are recruited to the site of infection, making its amount in the peripheral blood small (Antonelli et al., 2006). Gollob et al. (2008) suggests that these cells through the production of cytokines play a role associated with pathogenesis as well as plays a regulatory role in cutaneous leishmaniasis. In this study, there was no statistical significance regarding the percentage of these cells in the three groups of patients when compared with the group of healthy individuals. However, we observed a higher expression of CD45RO in these cells in the BT group, suggesting that these cells are activate when they are still in the peripheral blood and are recruited to the site of infection, and may contribute to the pathogenesis of the disease. In addition, there is a decrease in the expression of CD45RO in the SC group, raising the hypothesis that these cells can also act in the fight against infection or that theses patients did not had a substantial activation of their immune system. More markers will be needed to confirm these hypotheses in future studies.

5. Conclusion

In our study, we observed that CD4$^+$ T cells appear to be linked to protection and that CD8$^+$ T lymphocytes appear to have played a cytotoxic role, thus contributing to CL lesions. We observed a decrease of the CD45RO marker in the DP, DN and CD4$^+$ cells in subclinical patients, suggesting that a greater expression of CD45RO of these cells may be the mechanism responsible for the appearance of the lesions in patients with active CL. This marker appears to have a greater expression in effector cells than in memory cells and is thus more likely to be found in the active disease since, when recognizing the antigen, the lymphocytes enter into an activation process, where we see CD45RO expression, in order to counteract the infection.

The subclinical patients in our study appear to have had a Th1 response during infection since they did not present lesions at any time. We also observed that DP and DN lymphocytes, when expressing CD45RO, seem to play a dual role (pathogenesis and protection). We emphasize that more studies with a larger number of patients and more markers should be performed in order to characterize these lymphocyte populations.

Acknowledgments

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